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Rapid Communication

Epstein–Barr Virus-associated Gastric Adenocarcinoma

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The Epstein–Barr virus (EBV) has been detected in certain types of lymphoma and some epithelial neoplasms including nasopharyngeal lymphoepithelioma, and rare lymphoepithelioma-like carcinomas occurring in a variety of organs including, most recently, the stomach. The authors investigated the possibility that EBV may be present not only in the rare gastric cancers that resemble nasopharyngeal lymphoepithelioma, but also in typical gastric adenocarcinoma. EBV sequences were detected in 22 of 138 (16%) cases of typical gastric adenocarcinoma by polymerase chain reaction and* in situ *hybridization (ISH) techniques. The EBV genomes were specifically present within the gastric carcinoma cells in an even distribution. The EBV genomes were also present in adjacent dysplastic epithelium but were absent in surrounding lymphocytes, other normal stromal cells, intestinal metaplasia, and normal gastric mucosa. The EBV genomes in the infected gastric carcinoma cells are expressed as EBV RNA was detected by ISH. EBV was most often detected in gastric tumors from men (21%) compared with women (3%). Thus some cases of gastric adenocarcinoma are EBV-associated. (Am J Pathol 1992, 140:769–774)

The Epstein–Barr virus (EBV) infects oropharyngeal epithelium and B-lymphocytes, and the virus has been known for many years to be associated with nasopharyngeal lymphoepithelioma and certain B-cell lymphoproliferations such as endemic Burkitt's lymphomas and the lymphoproliferations that occur in immunosuppressed patients.[1] In the past few years, EBV has been identified in other lymphoid neoplasms, including Hodgkin's disease[2] and T-cell lymphoma.[3] However, with the exception of nasopharyngeal lymphoeptihelioma[4,5] and rare cases of lymphoepithelioma-like carcinomas occurring in other organs such as parotid gland,[6] thymus,[7,8] lung,[9] and stomach,[10–12] EBV has not been conclusively identified within the tumor cells of epithelial neoplasms.

In the course of study of a series of gastric lymphoepithelioma-like carcinomas,[12] we unexpectedly found a case of typical gastric adenocarcinoma that was positive for EBV. In the current report, we investigated the possibility that EBV may be present not only in the rare gastric cancers that resemble nasopharyngeal lymphoepithelioma, but also in typical gastric adenocarcinoma, using the polymerase chain reaction (PCR)[13] and *in situ* hybridization (ISH) techniques.

Methods

Specimens

Formalin-fixed, paraffin-embedded tissue blocks from 175 patients were studied; frozen tissue was not available for study. There were 138 patients with gastric adenocarcinoma specimens of which 67 were from surgical specimens (gastrectomy or biopsy) and 71 were from autopsies. The gastric adenocarcinomas were classified as intestinal or diffuse by the criteria of Lauren,[14] although tumors that did not fit the criteria for the intestinal classification were considered diffuse. None of the specimens resembled lymphoepithelial-like carcinomas. In addition, 10 specimens with Barrett's esophagus, 11 with esophageal squamous cell carcinoma, 8 with gastric ulceration, and 8 with normal gastric mucosa were studied as controls. With the exception of 18 gastric cancer specimens from the Hospital of the Good Samaritan of Los Angeles, all of the specimens were obtained from the files of the LAC-USC Medical Center. Demographic data

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were obtained from the pathology reports. Fisher's exact test with two-sided *P* values was used for statistical analysis.

The gastric carcinoma cell lines Kato III, HS 746T, AGS, and RF 48 were obtained from the American Type Culture Collection (Rockville, MD) and tested for the presence of EBV. All of these cell lines originated from male patients.

PCR

The specimens were first screened for EBV sequences by PCR. Ten micron thick sections were cut from formalin-fixed, paraffin-embedded tissues, and the DNA was extracted as previously described.[15] The PCR was completed as we have previously described[15] with primers (SL1, SL3) specific for an 80-base pair region of the EBV EBNA 1 gene. The authenticity of the PCR product was verified in each case by hybridization to an internal ^{32}P-labeled probe (SL2), and in some cases by restriction digest analysis with NcoI to produce the expected 53-base pair fragment. As a positive amplification control, primers (PC03, PC04[13]) for the human beta globin genomic sequence were also simultaneously present during the amplification. All EBV positive and negative samples showed amplification of the normal genomic sequence. Positive controls consisting of Raji DNA, and negative controls consisting of human genomic DNA as well as an assay with no added sample were completed with each experiment. The specificity and sensitivity of the methodology is reported elsewhere.[15]

Immunohistochemistry

The normal and tumor tissues were stained for keratin using a cocktail containing the four monoclonal antikeratin antibodies AE1 (Hybritech, San Diego, CA), CAM5.2 (Becton Dickinson, Mountain View, CA), UCD3 (Trition Biosciences, Alameda, CA), and EAB-902 (Enzo Biochemicals, New York, NY) using the avidin-biotin complex (ABC) method as previously described.[16]

In Situ *Hybridization*

Cases positive for EBV by PCR were studied by ISH to determine the cellular location of the EBV. The DNA ISH studies were performed as we have previously described using an ^{35}S-labeled *Bam*HI-W fragment of the EBV genome.[17] Previous negative hybridization studies with heterologous cytomegalovirus and papillomavirus probes showed the specificity of the *Bam*HI-W fragment for EBV sequences.[17] In each experiment, a fixed nasopharyngeal carcinoma specimen served as a positive control and EBV-negative lymphoid tissue served as a negative control.

The EBV RNA sequence, EBER1,[18] was detected with a complementary biotinylated 30-base oligomer using a procedure previously described.[19] Briefly, 10-μm sections cut from paraffin blocks of formalin-fixed tissues were deparaffinized, dehydrated, predigested with pronase, prehybridized, and hybridized overnight at a concentration of 0.25 ng/μl of probe. After washing, detection was accomplished using avidin-alkaline phosphatase conjugate followed by development of the signal with McGadey's substrate. A brown or blue-brown color within the nucleus over background levels was considered a positive reaction. This method detected EBV RNA from the EBV-infected Raji cell line, but not from the non–EBV-infected T-cell line Molt 3. In addition, cells from tissues that were infected with herpesvirus I, human papillomavirus 16, and adenovirus showed no crossreactivity. Although the sense strand oligonucleotide could not be used as a negative control (due to partial identity with adjacent antisense sequences), substitution of the probe with ten other oligonucleotides of identical length and similar G-C content showed no similar staining. Preincubation with 9 ug/μl of boiled ribonuclease A (Boehringer Mannheim, Indianapolis, IN) at 37°C overnight using buffer conditions recommended by the manufacturer, omission of the labeled probe, or addition of a 50-fold excess of unlabeled probe to the hybridization solution all caused elimination of the staining pattern. A known EBV-positive undifferentiated nasopharyngeal lymphoepithelioma served as a positive control and EBV-negative epithelial and lymphoid tissue served as negative controls for each assay. The dual immunohistochemical-RNA ISH studies were accomplished by performing the immunohistochemical assay first, followed by the RNA ISH assay, without modification of either procedure.

Results

EBV was detected in 22 of 138 (16%) cases of gastric adenocarcinoma (Figure 1, Table 1). This association was significant as EBV was not detected from any of the 37 control specimens with esophageal squamous cell carcinoma, Barrett's esophagus, gastric ulcer, or normal gastric mucosa ($P = 0.009$). The DNA and RNA ISH studies showed specific localization of the EBV genomes to the gastric carcinoma cells and in two of two cases to small adjacent foci of dysplastic epithelium (Figures 2–4). The surrounding lymphocytes, normal gastric mucosa, and intestinal metaplasia (present in some cases) were EBV negative. Within a given positive case, a uniform distribution of EBV genomes was found from neoplastic

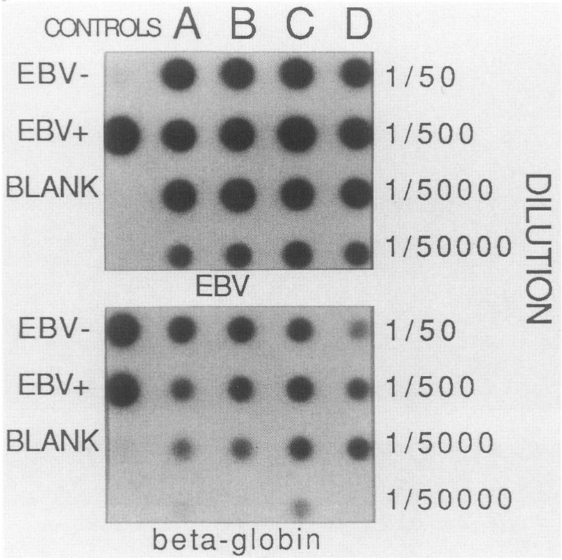


Figure 1. *Serial tenfold dilutions of the DNA extracted from four paraffin-embedded gastric cancers were amplified 50 cycles for both a genomic (beta-globin) and an EBV (EBV nuclear antigen 1) sequence. Equal amounts (10 µl) of PCR products were dotted onto two filters and hybridized against the beta-globin or EBV ^{32}P-labeled specific internal probes. The dilutions refer to relative amounts of DNA extracted from a single 10-µm section with "1" equivalent to the amount of DNA extracted from the entire section, "1/50" equivalent to 2% of the this amount, "1/500" equivalent to 0.2%, "1/5,000" equivalent to 0.02%, and "1/50,000" equivalent to 0.002%. The autoradiograph of the dot blots of the PCR products shows that large numbers of EBV sequences, comparable to the amounts genomic DNA, are present in the positive gastric cancer samples* **(A–D)**. *The controls consist of the PCR products amplified from 1,000 CEM (EBV−) or EB1 cells (EBV+, American Type Culture Collection, Rockville, MD), or no added sample (blank).*

cell to neoplastic cell. EBV sequences could also be detected in the metastatic carcinoma present in lymph nodes, lung, or liver in all ten EBV-associated carcinoma cases in which such tissues were available.

The EBV EBER1 sequence[18] is expressed and localized to the nucleus[20] of latently infected cells. Although the exact role of this small RNA is unknown, its high levels of expression (up to 10^7 copies per cell) facilitates detection by ISH. The EBER1 RNA was readily detected in the nuclei of the tumor cells, indicating that at least a portion of the EBV genome is transcribed in the infected gastric carcinoma cells (Figure 3).

Table 1. *Summary of EBV-associated Gastric Cancer*

	Number examined	EBV positive (%)
Gastric adenocarcinoma	138	22 (16)
Male	99	21 (21)
Female	39	1 (3)
Intestinal	95	15 (16)
Diffuse	43	7 (16)
Nongastric carcinoma*	37	0 (0)

* Esophageal squamous carcinoma (n = 11), Barrett's esophagus (n = 10), gastric ulcer (n = 8), normal gastric mucosa (n = 8).

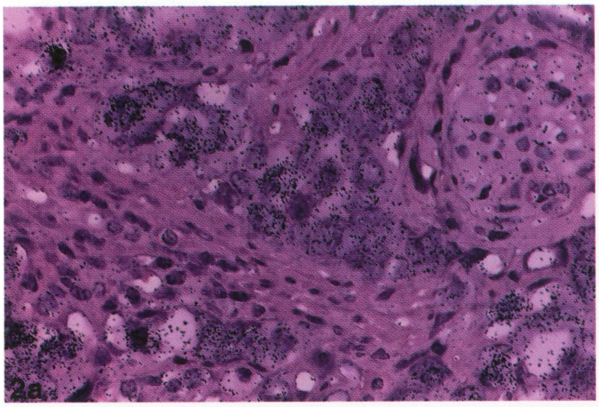
The EBV-associated cases were confirmed as carcinoma rather than lymphoma with the demonstration of keratin reactivity by immunohistochemical analysis. Additional confirmation of the specific EBV infection of the gastric carcinoma cells was obtained using dual immunohistochemical staining for keratin and ISH for EBV RNA (Figure 4). The EBV-associated gastric carcinomas were present in similar proportions (16%) of both intestinal-type tumors and diffuse-type tumors. The EBV-associated tumors occurred predominantly in the corpus or antrum. Colloid formation or large numbers of signet ring cells were not prominent features in any of the positive cases. Extensive lymphoid infiltration of the tumors was not present except for three cases that resembled the gastric carcinomas with intense lymphoid infiltration described by Watanabe et al,[21] and not lymphoepithelioma-like carcinomas. The EBV-associated cancers occurred predominantly in men (21 cases) compared with women (one case) ($P = 0.008$).

The gastric cancer cell lines Kato III, HS 756T, and AGS were EBV-negative by PCR. The RF 48-cell line was EBV positive by PCR. However, this cell line, which was established from the malignant ascites fluid in a patient with a poorly differentiated gastric primary,[22] was keratin negative and leukocyte common antigen (CD45) positive by immunohistochemical analysis (not shown), suggesting a lymphoid origin of this tumor.

Discussion

In the current study, three independent nucleic acid hybridization techniques, PCR, DNA ISH, and RNA ISH, complementary to three different regions of the EBV genome, demonstrated that EBV sequences are present in a subset of cases of gastric adenocarcinoma. Multiple EBV genomes were present in the gastric carcinoma cells based on the intensity of the DNA ISH signals and PCR dilution studies. In addition, at least one portion of the EBV genome is actively transcribed since the EBER1 gene,[18,20] which is one of seven EBV gene products expressed in latently infected cells, was readily detected by ISH in the nuclei of EBV-associated gastric carcinoma cells. In contrast, EBV could not be detected in the control nongastric cancers and non-neoplastic gastric tissues.

EBV could not be detected from three well-characterized gastric carcinoma cell lines. Study of more cell lines may detect positive cultures since only a minority of all gastric cancers are EBV-associated. Alternatively, similar to the EBV-negative epithelial cultures established from EBV-associated nasopharyngeal carcinoma tissues,[23] EBV sequences may be lost from the malignant gastric cells after passage *in vitro*.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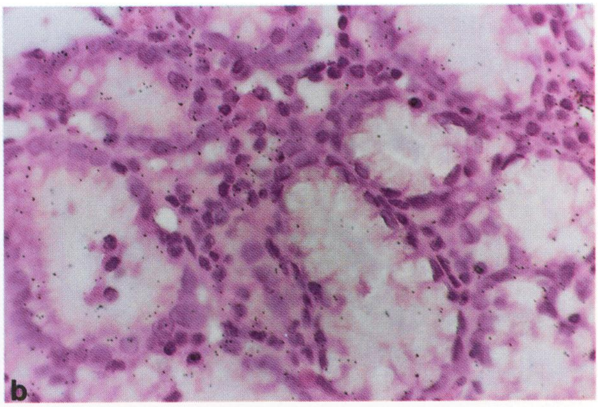
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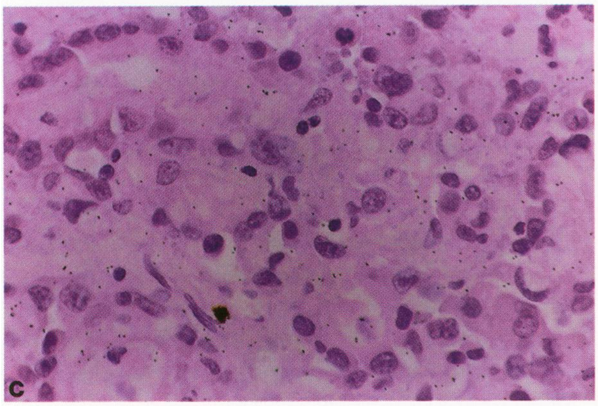
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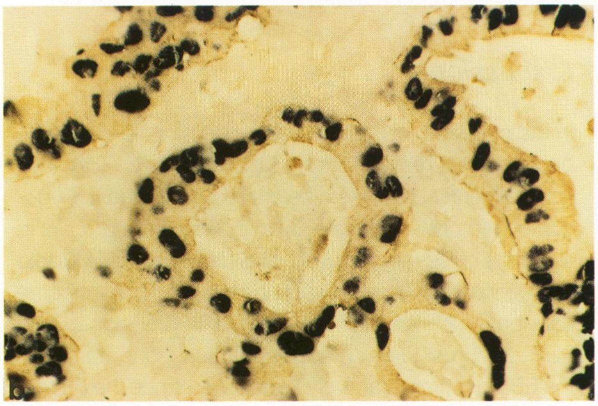
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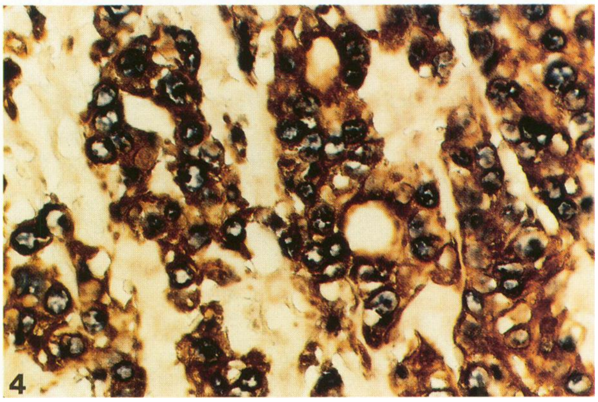
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Figure 2. *ISH for EBV DNA.* **A:** *Strong labeling of the carcinoma cells is present with only background levels of grains present over host lymphocytes, stromal cells, and the nerve present at the right of the lymphocytes, stromal cells, and the nerve present at the right of the field.* **B:** *No labeling is seen in the adjacent normal gastric mucosa.* **C:** *No labeling is seen in a gastric carcinoma that was negative for EBV by PCR (×600).*

Figure 3. *ISH for EBV RNA.* **(A)** *demonstrates that the neoplastic glands of the gastric carcinoma present to the left are positive for EBV RNA, whereas the stroma and the non-neoplastic glands to the right are negative (×100, no counterstain);* **(B)** *demonstrates that the EBV RNA is present in the nucleus whereas the cytoplasm and stroma remain unlabeled (×600, no counterstain);* **(C)** *deomonstrates no signal after hybridization with a nonsense oligonucleotide of similar length and G-C content (×100);* **(D)** *shows absence of signal in a gastric carcinoma that was negative for EBV by PCR (×600).*

Figure 4. *Dual detection of keratin by immunohistochemistry and EBV RNA by ISH. The nuclei stain blue-black, indicating the presence of EBV RNA, whereas the cytoplasm stains brown, indicating the presence of keratin and confirming the epithelial origin of the EBV-infected gastric adenocarcinoma cells (×600, no counterstain).*

The EBV-associated gastric cancers did not have distinct histologic features. Both intestinal type and diffuse type cancers were positive for EBV. Unlike poorly differentiated gastric lymphoepithelioma-like carcinomas that resemble nasopharyngeal carcinoma and are commonly EBV-positive[10–12] most of the EBV-associated gastric adenocarcinomas in this study lacked an intense lymphoid infiltration. Gastric carcinoma has a higher incidence in men[24] and the EBV-associated gastric cancers were present almost exclusively in men. The etiology of this sex bias is unknown. An X-linked lymphoproliferative syndrome[25] can result in fatal childhood EBV-associated infectious mononucleosis. A variant of this or another X-linked selective immunodeficiency may predispose some men to develop EBV-associated gastric cancer later in life. Interestingly, there is a marked excess of gastric cancers in patients with common variable immunodeficiency, who also are subceptible to EBV-associated lymphoproliferations.[26,27]

The biologic importance of the EBV genomes in the gastric cancers is debatable. The presence of EBV genomes and their expression in the gastric carcinoma cells raises the possibility that EBV may contribute to the neoplastic transformation. However, it is currently unknown when the EBV infection of the gastric epithelial cells occurs. The lack of detectable EBV sequences in non-neoplastic gastric epithelium suggests that an EBV receptor (CD21[28,29]) is not normally present on gastric epithelial cells. Indeed, the CD21 antibody B2 does not react with normal gastric epithelium, although gastric carcinoma, dysplasia, and intestinal metaplasia have not been studied (L. Weiss, unpublished observations). Therefore, EBV infection may occur after neoplastic transformation has induced aberrant expression of an EBV receptor, and the observed EBV-infection of gastric cancer may be an epiphenomenon. Such a post-transformation infection by a virus, however, would be unique since with previous examples of viruses associated with human tumors (such as HTLV-I, hepatitis B, papilloma virus), infection is believed to precede transformation and a considerable latent period occurs before transformation.

Alternatively, the gastric EBV infection may occur before and contribute to transformation. Our study showed that both the tumor and metastatic cells are uniformly EBV infected and therefore suggests that EBV infection occurs early in oncogenesis with a subsequent clonal expansion of EBV-containing tumor cells. Some changes in benign gastric mucosa associated with an increased risk for gastric cancer,[30] such as chronic gastric atrophy or intestinal metaplasia, may induce the expression of an EBV receptor and therefore allow infection to precede transformation. Further studies are needed to determine when in the course of neoplastic transformation the EBV infection occurs. Regardless of the timing of the EBV infection, its presence in these EBV-associated gastric cancers may facilitate their therapeutic management. For example, similar to patients with EBV-associated nasopharyngeal lymphoepithelioma,[31] elevated EBV antibody titers may serve as serologic markers for the early detection and follow-up of EBV-associated gastric carcinoma in high-risk populations. EBV-associated gastric cancers may also be vulnerable to antiviral therapy or immunotherapy against expressed viral antigens.

In summary, EBV genomes are present and expressed within the tumor cells of some gastric adenocarcinomas. Additional studies should clarify the role of EBV in the oncogenesis of gastric cancer.

Note Added in Proof

Southern blot analysis for EBV-fused termini[32] on an additional EBV-associated gastric adenocarcinoma demonstrated a single major band and a second very minor band, indicating a predominantly monoclonal proliferation of the EBV-associated carcinoma cells, which supports the hypothesis that EBV infection precedes transformation. Hybridizing bands less than 6 kb, diagnostic for viral replication,[33] were not present.

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